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# Regulation of G proteins by covalent modification

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**Heterotrimeric G protein α,β, and γ subunits are subject to several kinds of co- and post-translational covalent modifications. Among those relevant to G protein-coupled receptor signaling in normal cell function are lipid modifications and phosphorylation. *N*-myristoylation is a co-translational modification occurring for members of the $G_i$ family of Gα subunits, while palmitoylation is a post-translational modification that occurs for these and most other Gα subunits. One or both modifications are required for plasma membrane targeting and contribute to regulating strength of interaction with the Gβγ heterodimer, effectors, and regulators of G protein signaling (RGS proteins). Gα subunits, including those with transforming activity, are often inactive when unable to be modified with lipids. The reversible nature of palmitoylation is intriguing in this regard, as it lends itself to a regulation integrated with the activation state of the G protein. Several Gα subunits are substrates for phosphorylation by protein kinase C and at least one is a substrate for phosphorylation by the p21-activated protein kinase. Phosphorylation in both instances inhibits the interactions of these subunits with the Gβγ heterodimer and RGS proteins. Several Gα subunits are also substrates for tyrosine phosphorylation. A Gγ subunit is phosphorylated by protein kinase C, with the consequence that it interacts more tightly with a Gα subunit but less well with an effector.** *Oncogene* (2001) **20,** 1643–1652.

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Heterotrimeric G protein subunits are subject to a variety of covalent modifications, which occur in both normal and pathological contexts. With hardly an exception, G protein α (Gα) subunits undergo *N*-myristoylation and/or palmitoylation. G protein γ (Gγ) subunits are subject to prenylation. These lipid modifications in general are relevant to the targeting of subunits to membrane and to the interactions of these subunits with each other and other proteins. Some Gα subunits and a Gγ subunit are phosphorylated, a modification that appears to play a role in signal amplitude and duration. $G\alpha_s$ is ADP-ribosylated by cholera toxin, which prolongs its activation state, while most $G\alpha_i$ family members are ADP-ribosylated by a pertussis toxin (PTX), which disrupts interaction of these subunits with G protein-coupled receptors.

This review will cover three covalent modifications of mammalian G protein subunits – *N*-myristoylation, palmitoylation and phosphorylation. The reader is referred to several previous reviews of *N*-myristoylation and palmitoylation (Wedegaertner *et al*., 1995; Bhatnagar and Gordon, 1997; Mumby, 1997; Wedegaertner, 1998; Dunphy and Linder, 1998). Selected topics and advances in the last several years will be emphasized here. Prenylation and ADP-ribosylation will not be discussed. Prenylation is reviewed by Fu and Casey (1999).

## *N*-myristoylation and palmitoylation

We will focus on the contributions of *N*-myristoylation and palmitoylation to membrane targeting and subunit·protein interactions. The importance of these modifications to cell growth is highlighted in small part by the ability of mutations that prevent fatty acid acylation of certain Gα subunits to nullify the transforming activity of these subunits. Mutation of a constitutively active form of $G\alpha_{i2}$ to prevent *N*-myristoylation, for example, renders the subunit unable to transform Rat 1a fibroblasts (Gallego *et al*., 1992). A mutation preventing palmitoylation of a similarly active form of $G\alpha_{12}$ in NIH3T3 cells also prevents transforming activity (Jones and Gutkind, 1998).

### *Definition*

*N*-myristoylation represents the attachment of myristate (C14 : 0) through an amide bond to a glycine residue at the *N* terminus. The amide linkage is viewed in most instances to be irreversible. The reaction is catalyzed by myristoyl CoA : protein *N*-myristoyl transferase (NMT) (Johnson *et al*., 1994). NMT exhibits a strict requirement for $Gly^2$ (following cleavage of $Met^1$) and usually $Ser^6$ or $Thr^6$. The α subunits of the $G_i$ family, which contain a $Gly^2/Ser^6$ motif, are substrates for *N*-myristoylation, while α subunits of the $G_s$, $G_q$, and $G_{12}$ families are not. NMT catalyzes the attachment C14 : 0 almost exclusively, but is found under certain circumstances to attach C12 : 0, C14 : 1(Δ5), and C14 : 2(Δ5,8) (Bhatnagar and Gordon, 1997), as demonstrated for $G\alpha_t$ in the retinal rod cells (Neubert *et al*., 1992).

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Palmitoylation represents the attachment of palmitate (C16:0) through a thioester bond to a cysteine residue near (for Gα subunits) the *N* terminus. In contrast to *N*-myristoylation, palmitoylation is a reversible modification for which no clear consensus sequence has been identified. Palmitoylation can be achieved enzymatically (Berthiaume and Resh, 1995; Dunphy *et al*., 1996; Das *et al*., 1997), although a palmitoyl acyltransferase has not yet been purified to homogeneity, or non-enzymatically (Duncan and Gilman, 1996; Bañó *et al*., 1998). Depalmitoylation can be catalyzed by a cytoplasmic acyl-protein thioesterase both *in vitro* and in intact cells (Duncan and Gilman, 1998). Palmitate is usually the prevalent fatty acid incorporated, but alternatives can include stearate, arachidonate, and other long-chain fatty acids (O'Brien *et al*., 1987; Hallak *et al*., 1994b).

### *Targeting of $G\alpha_i$ family members to membrane*

One of the first roles accorded fatty acid acylation in the control of G protein function was membrane anchorage. Experiments with $G\alpha_i$ and $G\alpha_o$ prior to any knowledge of palmitoylation had shown that mutations preventing *N*-myristoylation (G2A) resulted in subunits unable to attach to membrane (Jones *et al*., 1990; Mumby *et al*., 1990). It was generally agreed that the hydrophobic nature of the myristoyl group might impart to the subunit an increased affinity for membrane. However, *N*-myristoylation could not be the whole story, since many G protein α subunits are not *N*-myristoylated yet are firmly attached to membrane. Moreover, the hydrophobicity imparted by the myristoyl group likely falls short of what is required for stable membrane attachment (Peitzsch and McLaughlin, 1993). It quickly became evident that, for α subunits of the $G_i$ family, *N*-myristoylation was a prerequisite to palmitoylation (Mumby *et al*., 1994; Hallak *et al*., 1994a; Galbiati *et al*., 1994), and that the palmitoyl moiety alone (and certainly in conjunction with the *N*-myristoyl moiety), was sufficient for attachment of at least peptides to membrane (Shahinian and Silvius, 1995).

Membrane anchorage of $G\alpha_i$ subunits conforms to a two-signal (lipid) membrane trapping model (Cadwallader *et al*., 1994; Shahinian and Silvius, 1995; Resh, 1996; Dunphy and Linder, 1998; Morales *et al*., 1998; Schroeder *et al*., 1996). According to this model, *N*-myristoylation supports a transient interaction of subunits with membrane, and those subunits encountering a membrane with the capacity to carry out palmitoylation are thus modified and become firmly attached and concentrated at this membrane. One membrane with a clearly identified capacity to carry out palmitoylation is the plasma membrane, due to selective enrichment in a protein acyltransferase (Dunphy *et al*., 1996) and/or palmitoyl-CoA. Thus, palmitoylation represents a mechanism for targeting $G\alpha_i$ family members to the plasma membrane. *N*-myristoylation does not appear to be *absolutely* required for palmitoylation or anchorage, as overexpression of βγ can support palmitoylation and anchorage of a nonmyristoylated subunit. In a physiologic setting, however, *N*-myristoylation plays a key role in palmitoylation and anchorage of $G\alpha_i$ family members at the plasma membrane.

When $G\alpha_z$, for example, is expressed in CHO cells, it targets rapidly to the plasma membrane coincident with palmitoylation (Morales *et al*., 1998; Fishburn *et al*., 1999). When mutated to prevent palmitoylation (C3A), it associates with intracellular membranes in addition to plasma membrane. When mutated to prevent *N*-myristoylation (G2A), it distributes between the cytosol and nucleus, and is not palmitoylated. Of interest is the capacity of Gβγ, when overexpressed, to redirect at least a portion of the G2A mutant to plasma membrane where it can be palmitoylated (Morales *et al*., 1998). Thus, Gβγ can serve essentially in the same capacity as *N*-myristoylation, though perhaps less effectively, under conditions of overexpression. The ability of overexpressed Gβγ to target a G2A mutant to membrane coincident with palmitoylation was also seen previously in studies with $G\alpha_{i1}$ (Degtyarev *et al*., 1994). It has been argued, as a consequence, that the geranylgeranyl group of the γ subunit in the Gβγ heterodimer can constitute the first lipid in the two-signal membrane trapping model when Gβγ is overexpressed, much the same as the *N*-myristoyl group of the α subunit does normally (Morales *et al*., 1998).

There is reason to believe that palmitoylation and interaction with Gβγ might play partly redundant roles in plasma membrane targeting of $G\alpha_i$ family members. In support of this contention, an *N*-myristoylated form of $G\alpha_z$ lacking palmitate (C3A), despite interacting with intracellular membranes, is still enriched in the plasma membrane, although not to the same extent as wildtype (Fishburn *et al*., 1999). Co-expression of βARK-ct, which sequesters Gβγ, decreases the extent to which this mutant, and wildtype, co-fractionate with plasma membrane. When Gβγ is artificially targeted to the outer membrane of mitochondria, $G\alpha_z$ and $G\alpha_z$C3A follow (Fishburn *et al*., 2000). This latter observation, in particular, suggests that Gβγ is a strong targeting signal. Perhaps, then, *N*-myristoylation serves not so much to provide a diffuse interaction of Gα subunits with membrane, but rather facilitates interaction of these subunits with Gβγ (Jones *et al*., 1990; Linder *et al*., 1991). Gβγ, in turn, would serve to anchor Gα subunits at the plasma membrane, which would be reinforced by subsequent palmitoylation. However, palmitoylation does not appear to require Gβγ. Several mutations in $G\alpha_o$ (e.g., deletion of residues 8–11 or insertion of 10 lysine residues between $Ser^6$ and $Ala^7$) disrupt interactions of the subunit with Gβγ but have no impact on palmitoylation (Wang *et al*., 1999b).

Of interest, when mutations that disrupt interactions of $G\alpha_o$ with Gβγ are combined with a G2A mutation to inhibit *N*-myristoylation, palmitoylation is no longer evident – even when plasma membrane targeting is apparently maintained (Wang *et al*., 1999b). Thus, *N*-

myristoylation would seem to play a role in palmitoylation beyond promoting interaction of $G\alpha_o$ with membrane or $G\beta\gamma$. To complicate the issue, $G\beta\gamma$ can also play a role in palmitoylation beyond interactions of $G\alpha_o$ with membrane. A mutation in $G\alpha_o$ that increases its affinity for $G\beta\gamma$ greatly enhances palmitoylation without overt changes in membrane association. *N*-myristoylation and $G\beta\gamma$, therefore, would appear to play roles in palmitoylation not only in terms of bringing the subunit to membrane but in post-anchorage events. Possible mechanisms are that the *N*-myristoyl moiety or $G\beta\gamma$ is recognized by a palmitoyltransferase as part of the substrate or that they position the *N* terminus of the $G\alpha$ subunit in such a manner as to facilitate palmitoylation. With respect to these roles, Dunphy *et al*. (1996) found that the palmitoylation of $G\alpha_i$ *in vitro* was enhanced by *N*-myristoylation and $G\beta\gamma$ independently. It is also probable that *N*-myristoylation and $G\beta\gamma$ protect $G\alpha_o$ against depalmitoylation. The role of *N*-myristoylation in this regard has been suggested previously (Degtyarev *et al*., 1994; Morales *et al*., 1998), and a role of this nature for $G\beta\gamma$ has precedent in studies with $G\alpha_s$ (Wedegaertner and Bourne, 1994; Mumby *et al*., 1994; Degtyarev *et al*., 1993b).

*N*-myristoylation, $G\beta\gamma$, and palmitoylation therefore play multiple, reinforcing roles in the targeting and anchorage of $G\alpha_i$ family subunits to plasma membrane (Figure 1). *N*-myristoylation alone promotes interactions of $G\alpha_i$ subunits with membrane by virtue of its (moderate) hydrophobicity. *N*-myristoylation additionally promotes interaction of $G\alpha_i$ subunits with $G\beta\gamma$; because $G\beta\gamma$ is independently anchored to membrane through prenylation, this strengthens the interaction of $G\alpha_i$ subunits with membrane. Immunocytochemical data suggest that $G\beta\gamma$ is not randomly distributed,

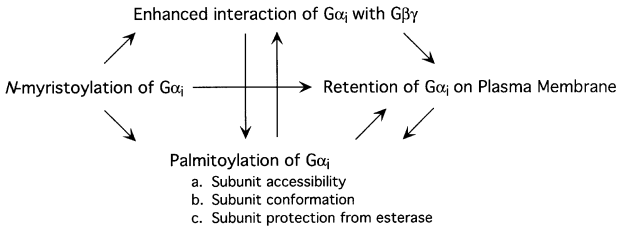


**Figure 1** Reinforcing roles of *N*-myristoylation, $G\beta\gamma$, and palmitoylation in targeting of $G\alpha_i$ to plasma membrane. *N*-myristoylation of $G\alpha_i$ enables the subunit to interact both with high affinity with $G\beta\gamma$ and reversibly with cellular membrane. The interaction with $G\beta\gamma$ can represent a targeting step, where the retention of the subunit on the plasma membrane is reinforced by palmitoylation. The interaction with cellular membrane achieved with the *N*-myristoyl moiety alone would lead to an increasing amount of palmitoylated subunit at the plasma membrane independent of $G\beta\gamma$, as random interactions of *N*-myristoylated $G\alpha_i$ with plasma membrane are followed by palmitoylation and retention. The palmitoylation supported by the direct or indirect membrane targeting functions alone of *N*-myristoylation and $G\beta\gamma$ are referred to in the figure as 'subunit accessibility'. *N*-myristoylation and $G\beta\gamma$ have additional functions, however, including their effects on $G\alpha_i$ conformation and protection of the palmitoylated subunit from acyl thioesterases

but rather is localized to plasma membrane (and Golgi). The promoted interaction of $G\alpha_i$ with $G\beta\gamma$, therefore, may constitute not only an anchorage but a targeting step. Interaction of $G\alpha_i$ with plasma membrane, through *N*-myristoylation alone or *N*-myristoylation-promoted $G\beta\gamma$ binding, would allow for palmitoylation, further trapping $G\alpha_i$ at the plasma membrane. *N*-myristoylation and $G\beta\gamma$ additionally promote palmitoylation through other functions, which may involve engaging a palmitoyltransferase, inducing a conformation suitable for palmitoylation, and/or protecting the subunit from a palmitoyl esterase. The actions of *N*-myristoylation and $G\beta\gamma$ at this level would appear to be redundant, but have not been well studied.

G protein subunits, including those of the $G\alpha_i$ family, can be found in specialized regions of the plasma membrane including caveolae and lipid rafts (reviewed by Anderson, 1998; Brown and London, 1998; Smart *et al*., 1999). Lipid rafts, which likely contribute to the structure of caveolae, are rich in sphingolipids and cholesterol, and are resistant to solubilization by detergents. Immunofluorescence and immunogold electron microscopy experiments using *en face* views of the inner side of the plasma membrane reveal a punctate and clustered distribution of $G\alpha_i$, respectively (for example Chang *et al*., 1994; Huang *et al*., 1997, 1999). Subcellular fractionation – involving buoyant-density centrifugation of Triton X-100- or sodium carbonate (pH 11)-extracted membranes (Chang *et al*., 1994; Lisanti *et al*., 1994; Song *et al*., 1997), of sonicated plasma membranes isolated from Percoll gradients (Smart *et al*., 1995), or of sheared plasma membranes isolated by an *in situ* silica-coating procedure (Schnitzer *et al*., 1995) – reveal co-fractionation of subunits with caveolin and/or low buoyant density membranes. Although the coincidence of $G\alpha_i$ and caveolin in membrane is not always compelling (Huang *et al*., 1997), *N*-myristoylation and palmitoylation would nevertheless appear to play roles in directing $G\alpha$ subunits to membrane fractions having low buoyant density (Song *et al*., 1997; Galbiati *et al*., 1999). Song *et al*. (1997) reported that approximately 35% of total wildtype $G\alpha_{i1}$ co-fractionated with caveolin in low buoyant density particles. Co-fractionation was reduced by 75% for a C3S mutant, and was undetectable for a G2A mutant. These results were corroborated in experiments with the *N* terminal domain of $G\alpha_{i1}$ attached to a green fluorescent protein (Galbiati *et al*., 1999). Here, complete targeting information was obtained with the dually acylated *N* terminal 32 residues of $G\alpha_{i1}$.

That targeting by fatty acylation relies at least in part on the lipid structure of rafts was demonstrated in studies with liposomes engineered to mimic rafts (sphingolipid- and cholesterol-rich liposomes (SCRL)) (Moffett *et al*., 2000). About 20% of *N*-myristoylated $G\alpha_i$, and about 50% of the *N*-myristoylated and palmitoylated subunit, reconstituted into SCRL. Similar values were obtained with liposomes that did not mimic rafts (phosphatidylcholine- and cholesterol-rich

liposomes (PC : Chol)), indicating that the *N*-myristoyl and palmitoyl moieties contribute in a relatively nonspecific manner to reconstitution efficiency. However, when SCRL containing the dually acylated subunit were extracted with Triton X-100, about 50% of the subunit was found associated with the Triton X-100-insoluble pellet, whereas only a marginal amount of subunit was found in the pellet from detergent extracted PC : Chol vesicles. This result suggests that the dually acylated subunit partitions into rafts. Interestingly, G$\beta\gamma$, which is geranylgeranylated, does not partition into these rafts, and dually acylated G$\alpha_i$ when introduced as a heterotrimer with G$\beta\gamma$ displays reduced partitioning, suggesting that the geranylgeranyl moiety is not sufficient for partitioning and, moreover, that it may exert a dominant effect on the partitioning of dually acylated G$\alpha_i$. Differences between mono- and dual-acylation were not examined. Unsaturated fatty acids (e.g., 16 : 1) in place of palmitate resulted in less resistance to Triton X-100 extraction.

### *Targeting of other Gα subunits to membrane*

The majority of G protein $\alpha$ subunits are not *N*-myristoylated, but are palmitoylated as apparently the sole fatty acid modification. Some are palmitoylated at one site (G$\alpha_s$ and G$\alpha_{12}$) and others at potentially two sites (G$\alpha_q$ and G$\alpha_{13}$), but in all cases the modification occurs near the *N* terminus. While it is assumed that palmitoylation represents a targeting mechanism, it is less clear that it is required for stable membrane anchorage. Some investigators find that mutations preventing palmitoylation have little effect on anchorage (Degtyarev *et al*., 1993a; Mumby *et al*., 1994; Hepler *et al*., 1996; Jones and Gutkind, 1998), while others find that a similar mutation or a G protein activation event leading to depalmitoylation causes release of the subunits into cytosol (Wedegaertner *et al*., 1993, 1996; Wise *et al*., 1997; Bhattacharyya and Wedegaertner, 2000).

If *N*-myristoylation is essential to palmitoylation of G$\alpha_i$ subunits, which is a tenet of the two signal-trapping hypothesis, what then supports palmitoylation of G$\alpha$ subunits that are not *N*-myristoylated? One possible answer is an additional lipid modification yet to be identified. G$\alpha_s$ purified from rabbit liver stimulates membrane-bound adenylyl cyclase with an $EC_{50}$ of about 0.1 nM, while the $EC_{50}$ for G$\alpha_s$ expressed and purified from *E. coli* is 50 nM (Kleuss and Gilman, 1997). The difference in values may be related to a co- or post-translational modification unique to the mammalian subunit that enhances interaction of G$\alpha_s$ with membrane or with adenylyl cyclase directly. The modification is probably not palmitoylation, as the difference in $EC_{50}$ values was still observed following treatment of the subunits with hydroxylamine. The difference was lost, however, following removal of approximately the first 30 residues of the subunits with a protease. Differential partitioning of the two forms of subunit in Triton X-114 at 20°C indicated that the putative modification of the mammalian subunit is hydrophobic; G$\alpha_s$ from rabbit liver partitions into the detergent-rich phase, while G$\alpha_s$ from *E. coli* partitions into the aqueous phase. A similar modification is suspected for G$\alpha_q$ (Hepler *et al*., 1996). Purified mammalian G$\alpha_q$ partitions into the detergent phase, but so does a portion of the non-palmitoylated C9S/C10S mutant and palmitoylthioesterase-treated wildtype subunit. Whether the suspected modification is sufficient to direct membrane binding and palmitoylation is unclear.

Evanko *et al*. (2000), meanwhile, have provided good arguments that G$\beta\gamma$ is a required signal for membrane anchorage and palmitoylation of G$\alpha_s$ and G$\alpha_q$. Using point mutations, they demonstrated that mutants of these two subunits unable to bind G$\beta\gamma$ assumed an apparently cytosolic location and were not palmitoylated. Restoration of plasma membrane targeting and palmitoylation to a mutant of G$\alpha_q$ was achieved by an A2G mutation to enable *N*-myristoylation; the *N*-myristoyl moiety did not restore interaction of the mutant with G$\beta\gamma$. Thus, for G$\alpha_s$ and G$\alpha_q$, G$\beta\gamma$ would appear to be the functional equivalent of *N*-myristoylation. One implication of these studies is that members of the G$\alpha_i$ family do not interact sufficiently well with G$\beta\gamma$ in the absence of an *N*-myristoyl moiety to use G$\beta\gamma$ alone for anchorage.

### *Protein interactions facilitated by lipid modifications*

Lipid modifications clearly have roles beyond membrane targeting. The first of these to be realized was an increase in affinity of G$\alpha_i$ family members for G$\beta\gamma$ promoted by *N*-myristoylation (Jones *et al*., 1990; Linder *et al*., 1991). The magnitude of this increase in affinity is considerable: without the *N*-myristoyl (and palmitoyl moiety), G$\alpha_o$ does not bind stably to G$\beta\gamma$, nor does G$\beta\gamma$ interact sufficiently well with G$\alpha_o$ to suppress GDP dissociation (Linder *et al*., 1991). Both actions can be restored by an *N*-myristoyl moiety alone. Interactions between nonmyristoylated G$\alpha_i$ family subunits and G$\beta\gamma$ can nevertheless be detected by the ability of G$\beta\gamma$ to support PTX-catalyzed ADP-ribosylation of nonmyristoylated G$\alpha_i$, however this ability is also decreased (several-fold) compared to that for the *N*-myristoylated subunits (Jones *et al*., 1990; Linder *et al*., 1991). Consistent with some ability of a nonmyristoylated G$\alpha_i$ to interact with G$\beta\gamma$, targeting and palmitoylation of a nonmyristoylated subunit is restored by overexpressed G$\beta\gamma$ (Degtyarev *et al*., 1994; Morales *et al*., 1998).

Palmitoylation helps to support interactions of G$\alpha_s$ with G$\beta\gamma$ (Iiri *et al*., 1996). Whether the effects are as profound as those determined for *N*-myristoylation of G$\alpha_i$ family members has not been assessed.

Not only may lipid modifications of G$\alpha$ subunits influence protein interactions involved in limiting their signal (i.e., G$\beta\gamma$ binding), they may also play a role in mediating interactions that are conducive to transduction of signal, for example by promoting binding to

effectors or by inhibiting association with GTPase activating proteins. There are only a few examples of this to date. *N*-myristoylation of $G\alpha_i$ appears to be required for inhibition of adenylyl cyclase *in vitro* (Taussig *et al*., 1993), since *N*-myristoylated recombinant $G\alpha_i$ from *E. coli* inhibited adenylyl cyclase activity in Sf9 membranes upon activation, whereas nonmyristoylated $G\alpha_i$ did not. Interaction with adenylyl cyclase directly versus indirectly through membrane binding was not evaluated, however. Consistent with these data, constitutively active nonmyristoylated $G\alpha_i$ did not regulate adenylyl cyclase when expressed in Rat 1a cells, despite association with membrane, although the contributions of the loss of palmitoylation and potential mistargeting were not assessed (Gallego *et al*., 1992). Nonpalmitoylated mutants of $G\alpha_q$ (C9,10A or C9,10S) have a reduced ability to activate phospholipase-$\beta$ *in vitro*, however $G\alpha_q$ that was palmitoylation-deficient as a result of treatment with an esterase was not affected, suggesting a role for the cysteine residue itself (Hepler *et al*., 1996). Recent data for $G\alpha_i$ family subunits suggest an interplay between palmitoylation and the GTPase activity promoted by RGS proteins (Tu *et al*., 1997). While palmitoylation of purified $G\alpha_z$ had no effect on its intrinsic rate of GTP hydrolysis, palmitoylation inhibited its ability to respond to RGS proteins. The affinity of $G_z$ GAP for palmitoylated $G\alpha_z$ was reduced as compared to that for nonpalmitoylated $G\alpha_z$. Additionally, the maximal rate of GTP hydrolysis promoted by $G_z$ GAP was reduced for the palmitoylated subunit. Both the affinity and GTPase activity were restored upon removal of palmitate by dithiothreitol. Similar results were obtained with $\alpha_{i1}$ and RGS4.

### *Dynamics of palmitoylation*

Due to the reversible nature of palmitoylation, it has been viewed over the years as a potential site for modulating G protein function. Changes in the palmitoylation status of Gα may be particularly important, or even required, for enabling specific protein interactions and subcellular localization of the subunit. In this capacity, dynamic palmitoylation could provide both a temporal and spatial regulation of G protein mediated signals. How might palmitoylation be regulated? One possibility that has yet to be explored is modulation, upon specific stimuli, of the enzymes that catalyze palmitate turnover. Despite rigorous efforts, very little is known about a potential palmitoyltransferase (Berthiaume and Resh, 1995; Dunphy *et al*., 1996; Das *et al*., 1997) that is specific for Gα, therefore whether this enzyme itself is regulated remains to be determined. Characterization of a thioesterase that depalmitoylates Gα *in vitro* and in intact cells (Duncan and Gilman, 1998) is currently underway. Superimposed on the potential regulation of enzymes that catalyze turnover of palmitate is the possibility that palmitoylation is modulated by the activation state of Gα, due to specific conformations and/or accessibility to necessary enzymes. Indeed, it has been shown that the palmitoylation/depalmitoylation cycle is accelerated following activation of $G\alpha_s$ (Degtyarev *et al*., 1993b; Mumby *et al*., 1994; Wedegaertner and Bourne, 1994), $G\alpha_i$ (Bhamre *et al*., 1998; Stanislaus *et al*., 1998; Chen and Manning, 2000), and possibly $G\alpha_q$ (Gurdal *et al*., 1997; Stanislaus *et al*., 1997; Bhamre *et al*., 1998).

The dynamics of palmitoylation were first documented for $G\alpha_s$ and have been discussed at great length (Milligan *et al*., 1995; Ross, 1995; Wedegaertner *et al*., 1995; Mumby, 1997). An increase in [$^3$H]palmitate incorporation was demonstrated by several investigators following activation of $G\alpha_s$ by the $\beta_2$-adrenergic receptor (Degtyarev *et al*., 1993b; Mumby *et al*., 1994; Wedegaertner and Bourne, 1994) and cholera toxin (Degtyarev *et al*., 1993b), presumably due in part to an increase in depalmitoylation, facilitating an exchange of palmitate for [$^3$H]palmitate. When depalmitoylation was more directly measured by release of [$^3$H]palmitate in pulse-chase assays, an increase was also observed upon activation of $G\alpha_s$ through the $\beta_2$-adrenergic receptor (Wedegaertner and Bourne, 1994; Mumby *et al*., 1994) and by inhibition of GTPase activity by mutagenesis (Wedegaertner and Bourne, 1994). Recently it has been shown that for a $\beta_2$-adrenergic receptor·$G\alpha_s$ fusion protein, the extent of $G\alpha_s$ depalmitoylation induced by a series of agonists correlated with their intrinsic efficacy to stimulate adenylyl cyclase (Loisel *et al*., 1999). Additionally, while depalmitoylation remained activation-dependent despite the lack of receptor desensitization/internalization, the incorporation of palmitate was inhibited. These data indicate that complete dissociation of activated Gα from ligand-bound receptor is not strictly required for depalmitoylation. Furthermore, they suggest that while sustained activation allows for depalmitoylation, it may limit the repalmitoylation reaction. Alternatively, events subsequent to receptor internalization may be required for repalmitoylation.

The concept of regulated palmitate exchange has recently been extended beyond $G\alpha_s$. Palmitoylation of $G\alpha_i$ is subject to regulation upon its activation by a G protein-coupled receptor (Chen and Manning, 2000). In this case, the 5-$HT_{1A}$ receptor was demonstrated to promote palmitate exchange on endogenous $G\alpha_i$ in CHO cells through the combined processes of depalmitoylation and palmitoylation, as seen for $G\alpha_s$. Incorporation and pulse-chase experiments with [$^3$H]palmitate demonstrated a dose- and time-dependent change in radiolabeling of $G\alpha_i$ upon activation of the 5-$HT_{1A}$ receptor by the agonist 8-OH-DPAT. These changes were specific to receptor stimulation and receptor·$G_i$ coupling, as they were inhibited by the antagonist MPPI and the bacterial toxin PTX, respectively. Increases in incorporation of [$^3$H]palmitate for $G\alpha_i$ may also occur in pituitary cells following activation of gonadotropin-releasing hormone receptor (Stanislaus *et al*., 1998), and in rat brain membranes *in vitro* following activation of serotonin receptors (Bhamre *et al*., 1998). Curiously, agonist stimulation of the $D_2$ dopamine receptor in CHO-K1 cells did not

promote depalmitoylation of wildtype epitope-tagged $G\alpha_z$, another member of the $G_z$ family (Morales *et al*., 1998). This difference between $G\alpha_i$ and $G\alpha_z$ may be related to receptor expression and receptor coupling efficiencies, or regulated palmitate turnover may not occur for all $G_i$ family $\alpha$ subunits. Worth noting, a substantial increase in the rate of depalmitoylation was observed for a nonmyristoylated $G\alpha_z$ mutant (G2A) brought to membrane by overexpressed $\beta\gamma$, and this increase was further promoted by agonist, suggesting that the presence of the myristoyl moiety in wildtype $G\alpha_z$ significantly slows its depalmitoylation. The current model for activation-dependent depalmitoylation favors that upon dissociation of $G\alpha$ from $G\beta\gamma$, $G\alpha$ is more susceptible to an esterase that removes palmitate (Wedegaertner and Bourne, 1994; Duncan and Gilman, 1998). Perhaps, the significantly slow rate of depalmitoylation for $G\alpha_z$ may be partially explained by its slow rate of GDP/GTP exchange and thus $\alpha{\cdot}\beta\gamma$ dissociation, in addition to possibly its tight binding to $\beta\gamma$ conferred by *N*-myristoylation.

Does regulated palmitate exchange occur for members of the $G_q$ and $G_{12}$ families? Data for $G\alpha_q$ suggest that it does, while studies on $G\alpha_{12}$ and $G\alpha_{13}$ are yet to be carried out. For $G\alpha_q$, incorporation of [$^3$H]palmitate increases upon gonadotropin-releasing hormone receptor activation in pituitary cells (Stanislaus *et al*., 1997), however the potential effect on subunit synthesis needs further evaluation. Incorporation of [$^3$H]palmitate into $G\alpha_q$ also increases upon serotonin receptor activation in rat brain membranes *in vitro* (Bhamre *et al*., 1998), and upon $\alpha$-adrenergic receptor activation in aortic membranes *in vitro* (Gurdal *et al*., 1997).

The status of $G\alpha$ palmitoylation may contribute positively or negatively to signal transduction. For $G\alpha_s$, a model has been proposed in which regulated depalmitoylation upon activation leads to translocation of the subunit from plasma membrane to cytosol, thus limiting the proximity to membrane-bound effectors and dampening signal (Wedegaertner and Bourne, 1994; Wedegaertner *et al*., 1996). Another model suggests that depalmitoylated $G\alpha_s$ remains at the plasma membrane, and that activated subunits concentrate in subdomains (Huang *et al*., 1999), possibly either enhancing or limiting signal in this manner. This model does not preclude, however, that depalmitoylation leads to a translocation event in that one could envision, among many other possibilities, that active and palmitoylated subunits are targeted to distinct membrane domains, where depalmitoylation then occurs, leading to translocation of the subunit back to the membrane proper. The latter model may better suit $G\alpha_i$, in that depalmitoylation upon activation (our unpublished results) and depalmitoylation by recombinant esterase (Huang *et al*., 1999) do not appear to release the subunit into the cytosol. Alternatively or in addition, for $G\alpha_i$, depalmitoylation may dampen signal through promoting RGS interactions and RGS GAP activity, as suggested (Tu *et al*, 1997).

## Phosphorylation

### *Serine phosphorylation*

#### *PKC*

$G\alpha_i$ family: $G\alpha_z$ was among the first G protein subunits to be identified unequivocally as a substrate for phosphorylation, and in particular for the reaction catalyzed by protein kinase C (PKC). Incubation of human platelets with phorbol ester, which activates classical forms of PKC directly, resulted in a rapid phosphorylation of $G\alpha_z$ (Carlson *et al*., 1989). Phosphorylation was also achieved with thrombin and U46619, which activate PKC indirectly through phosphoinositide hydrolysis. As determined by phosphoamino acid analysis and cyanogen bromide peptide mapping, the site of phosphorylation in platelets was constrained to one or more serine residues in the *N* terminal 53 amino acids (Lounsbury *et al*., 1991). That an antibody directed toward $R^{24}SESQRNRRE^{33}$ was sensitive to phosphorylation indicated that $Ser^{25}$ or $Ser^{27}$ was the modified residue, and the almost complete abrogation of immunoreactivity in response to PMA indicated a stoichiometry of at least one mol phosphate per mol subunit. Subsequent studies with mutants of $G\alpha_z$ expressed in HEK293 cells revealed $Ser^{27}$ to be the preferred site of phosphorylation, and $Ser^{16}$ to be a secondary site; a small amount of phosphorylation was evident elsewhere (Lounsbury *et al*., 1993). None of the other subunits examined ($G\alpha_s$, $G\alpha_i$, and $G\alpha_q$) were found to be phosphorylated in these studies.

Evaluation of phosphorylation with PKC and $G\alpha$ subunits *in vitro* confirmed the stoichiometric nature and selectivity of phosphorylation (Lounsbury *et al*., 1991). However, whereas this study suggested a stoichiometry approaching 1 mol phosphate per mol subunit, a more recent study suggested a stoichiometry of two, probably representing complete phosphorylation of both $Ser^{16}$ and $Ser^{27}$ (Wang *et al*., 1999a). Time-courses of phosphorylation of S16A and S27A mutants indicated a kinetic preference for $Ser^{27}$.

There is some debate as to whether phosphorylation of $G\alpha_z$ is sensitive to the activation state of the subunit. In the earliest study, which used recombinant $G\alpha_z$ purified from *E. coli*, phosphorylation occurred preferentially for the GDP-bound form of subunit; GTP$\gamma$S suppressed phosphorylation by about 70% at early, though not later, time points (Lounsbury *et al*., 1991). In two other studies, with recombinant $G\alpha_z$ purified from Sf9 cells, phosphorylation was unaffected by GTP$\gamma$S (Kozasa and Gilman, 1996) or $AlF_4^-$ (Wang *et al*., 1999a). The differences in results might be attributable to differences in subunit processing, e.g., *N*-myristoylation and palmitoylation occur in Sf9 cells but not bacteria, or to the time point examined. All agree, however, that the monomeric form of $G\alpha_z$ is the preferred substrate for PKC, as $G\beta\gamma$ markedly suppresses phosphorylation (Fields and Casey, 1995; Kozasa and Gilman, 1996; Wang *et al*., 1999a), This observation is not surprising, as $G\beta\gamma$ binds the *N* terminal domain (among other regions) of

Gα subunits and would therefore hinder PKC stearically.

Phosphorylation, in turn, blocks Gβγ binding. The blockade was demonstrated by chromatography (Fields and Casey, 1995), sucrose density centrifugation (Fields and Casey, 1995), gel filtration (Kozasa and Gilman, 1996), and measurements of GDP/GTPγS exchange (Kozasa and Gilman, 1996; Wang *et al*., 1999a). Phosphorylation also inhibits interaction of $G\alpha_z$ with the RGS proteins RGSZ1, RET-RGS1, and GAIP (Glick *et al*., 1998; Wang *et al*., 1998). In terms of function, inhibition of interactions with Gβγ and RGS proteins might prolong the activation of $G\alpha_z$ (Figure 2); phosphorylation has little or no effect on the ability of $G\alpha_z$-GTPγS to inhibit the effector adenylyl cyclase (Kozasa and Gilman, 1996).

Data for other members of the $G\alpha_i$ family do not yet provide a consistent story. Some reports indicate that $G\alpha_i$ or $G\alpha_t$ can be phosphorylated directly by PKC (Katada *et al*., 1985; Zick *et al*., 1986; Daniel-Issakani *et al*., 1989) or in response to PMA treatment of cells (Bushfield *et al*., 1990; Strassheim and Malbon, 1994). Others indicate that it cannot (Carlson *et al*., 1989; Lounsbury *et al*., 1991; Kozasa and Gilman, 1996). To some extent, these differences may relate to assay conditions or the type of cell being analysed.

$G\alpha_{12}$ and $G\alpha_{13}$: $G\alpha_{12}$, like $G\alpha_z$, is unequivocally a substrate for PKC. $G\alpha_{12}$ introduced into NIH3T3 cells is phosphorylated following exposure of the cells to PMA (Kozasa and Gilman, 1996), and $G\alpha_{12}$ present endogenously in human platelets is phosphorylated in response to PMA, thrombin, and U46619 (Offermanns *et al*., 1996). The phosphorylation can be achieved *in vitro* with purified PKC and subunit, and proceeds to about 1 mol phosphate per mol subunit at least with PKCα (Kozasa and Gilman, 1996). The phosphorylation occurs within the *N* terminal 50 residues, but has not been mapped further. The context of $Ser^{38}$ in $G\alpha_{12}$, however, strongly resembles that of $Ser^{16}$ in $G\alpha_z$. As with $G\alpha_z$, Gβγ blocks phosphorylation, and phosphorylation reciprocally blocks interaction of the subunit with Gβγ.

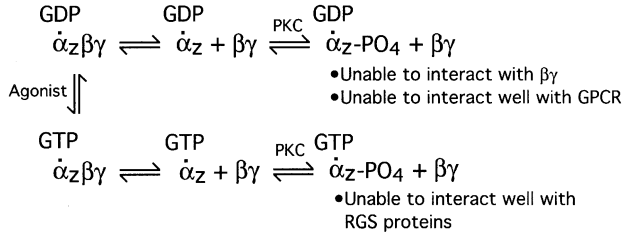


**Figure 2** The known or probable effects of PKC-mediated phosphorylation on the function of $G\alpha_z$. The PKC-mediated phosphorylation of $G\alpha_z$, which takes place near the *N* terminus and may occur for either the GDP- or GTP-liganded form of subunit, clearly inhibits the ability of the subunit to interact with Gβγ and RGS proteins. The disrupted interaction with Gβγ may adversely affect formation of high-affinity ternary complexes involving G protein-coupled receptors

The potential of $G\alpha_{13}$ to be phosphorylated by PKC is less clear. *In vitro* experiments with purified $G\alpha_{13}$ and PKC suggest that the subunit is not a substrate for PKC; $G\alpha_z$ and $G\alpha_{12}$ were the only substrates for phosphorylation in these experiments regardless of PKC isozymes employed (α, δ, ε, or ζ) (Kozasa and Gilman, 1996). Studies with platelets, however, demonstrated that $G\alpha_{13}$ is phosphorylated in response to PMA, and phosphorylation of $G\alpha_{13}$ expressed in COS cells was dependent on co-expression of PKC, where β, δ, and ε isozymes were most effective (Offermanns *et al*., 1996). The discrepancy between *in vitro* and intact cell experiments might be accounted for by unsatisfied requirements on the part of $G\alpha_{13}$ *in vitro* for PKC-mediated phosphorylation, e.g. subunit conformation or ancillary factors, though conditions were suitable for phosphorylation of $G\alpha_{12}$ and $G\alpha_z$. Alternatively, one or more kinases may be positioned between PKC and $G\alpha_{13}$ in the intact cell.

$G\gamma_{12}$: $G\gamma_{12}$ is also a substrate for PKC *in vitro* and in intact cells. $G\gamma_{12}$, which alone among Gγ subunits contains a SSK motif at the *N* terminus, is phosphorylated to about 1 mol phosphate per mol subunit *in vitro* by PKCα and β (Morishita *et al*., 1995; Yasuda *et al*., 1998), less well by δ and ε, and not at all by ζ (Morishita *et al*., 1995). Phosphorylation was also achieved with PMA for $G\gamma_{12}$ endogenous to Swiss 3T3 fibroblasts. The first serine of the SSK motif is proposed to be the site of phosphorylation. Phosphorylation increased the affinity of $G\beta\gamma_{12}$ for $G\alpha_o$ (and $G\alpha_i$) to some extent, as determined by affinity chromatography and enhancement in PTX-catalyzed ADP-ribosylation (Morishita *et al*., 1995). The formation of a more stable heterotrimer may account for the increase in potency (several-fold) of phosphorylated $G\beta\gamma_{12}$ in supporting high-affinity agonist binding to receptor (Yasuda *et al*., 1998). Phosphorylation in this latter study was also noted to have an effect on $G\beta\gamma_{12}$ interaction with an effector, adenylyl cyclase, as shown by inhibition (a doubling of $K_{act}$) of $G\beta\gamma_{12}$-mediated stimulation of adenylyl cyclase type II. However, the phosphorylation had no impact on activation of phospholipase C-β, indicating selectivity in the effect of phosphorylation on effector interactions. $G\gamma_{12}$ thus far is unique among γ subunits in interacting with F-actin, and its phosphorylation has been argued to enhance fibroblast motility through changes in actin filament assembly/disassembly (Ueda *et al*., 1999). A doubling of NIH3T3 cell motility was demonstrated following overexpression of $G\gamma_{12}$, which undergoes some level of basal phosphorylation, and $G\gamma_{12}(S\rightarrow E)SK$, which resembles a phosphorylated $G\gamma_{12}$, but not $G\gamma_{12}\Delta N5$ or $G\gamma_{12}S2A$, both of which lack the capacity to be phosphorylated.

*PAK* $G\alpha_z$ is a substrate for phosphorylation not only by PKC, but by the p21-activated protein kinase PAK (Wang *et al*., 1999a). *In vitro*, PAK1 catalyzed phosphorylation of recombinant $G\alpha_z$ at $Ser^{16}$, achieving a stoichiometry of about 1 mol phosphate/mol subunit.

Activation of $G\alpha_z$ with $AlF_4^-$ had no effect on phosphorylation. None of the other subunits tested ($G\alpha_s$, $G\alpha_i$, $G\alpha_o$, and $G\alpha_q$) were substrates. Phosphorylation of $G\alpha_z$ in HEK293 cells upon co-transfection with PAK1 (and activated Rac1) proceeded to the same extent as that of $G\alpha_z$ with PKC. The effects of PAK1-mediated phosphorylation on interactions of $G\alpha_z$ with $G\beta\gamma$ and RGS proteins were much the same as those of PKC-mediated phosphorylation. $G\beta\gamma$ inhibited phosphorylation of $G\alpha_z$ by PAK1, however the mechanism was not limited to blocking the site of phosphorylation – $G\beta\gamma$ inhibited the ability of PAK1 to phosphorylate other substrates, for example myelin basic protein and MEK, indicating an effect on the enzyme itself. Unusually, the inhibitory actions of $G\beta\gamma$ were exerted whether or not the heterodimer was free or complexed to $G\alpha$ subunits or phosducin.

### *Tyrosine phosphorylation*

Early work suggested that some $G\alpha_i$ family members are phosphorylated on tyrosine residues *in vitro* by the insulin receptor kinase (Zick *et al*., 1986; Krupinski *et al*., 1988). More data exist for phosphorylation of $G\alpha$ subunits by nonreceptor tyrosine kinases. Phosphorylation of at least several $G\alpha$ subunits ($G\alpha_s$ and $G\alpha_i$ family members) is achieved *in vitro* with $pp60^{c\text{-}src}$ with a stoichiometry of 0.3 – 0.9 mol phosphate per mol subunit (Hausdorff *et al*., 1992). $G\beta\gamma$ and $GTP\gamma S$ inhibit phosphorylation, suggesting a preference for the inactive monomeric $G\alpha$ subunit. Phosphorylation of $G\alpha_s$, which occurs on $Tyr^{37}$ and $Tyr^{377}$ (Moyers *et al*., 1995), enhances the capacity of $G_s$ to be activated in phospholipid vesicles by the $\beta_2$-adrenergic receptor. Transformation of fibroblasts with the v-*src* oncogene results in a several-fold enhancement of endothelin-1-stimulated inositol 1,4,5 trisphosphate accumulation, a process normally involving a member of the $G_q$ family (Liu *et al*., 1996). Immunoblotting with a phosphotyrosine-specific antibody revealed that $G\alpha_{q/11}$ (the two are not easily distinguished) was tyrosine phosphorylated in the transformed cells. Comparison of $G\alpha_{q/11}$ activities in detergent extracts reconstituted with PLC *in vitro* showed a twofold increase in $AlF_4^-$-stimulated activity for the phosphorylated subunit(s). While the phosphorylation was inhibited by herbimycin A, the authors commented that attempts to demonstrate a direct phosphorylation of $G\alpha_{q/11}$ with $pp60^{v\text{-}src}$ were unsuccessful, indicating that a kinase downstream of $pp60^{v\text{-}src}$ might be involved.

Tyrosine phosphorylation of $G\alpha_q$ and $G\alpha_{11}$ in the context of agonist signaling has also been described, wherein carbachol stimulation through an M1 muscarinic receptor stimulates tyrosine phosphorylation of the two subunits (Umemori *et al*., 1997). The site of phosphorylation appears to be the fourth residue from the C terminus (analogous to $Tyr^{377}$ of $G\alpha_s$), based on the inability of $G\alpha_{11}$ Y356F to be tyrosine phosphorylated. The sequence of events is not clear but the carbachol-promoted tyrosine phosphorylation can be mimicked by Fyn. The site of phosphorylation suggests an effect on coupling of the $G\alpha$ subunit to receptor, and some evidence for this was presented based on changes in agonist binding.

### *Histidine phosphorylation*

$G\beta$ has been demonstrated to be phosphorylated (or thiophosphorylated) by an enzyme that utilizes GTP (or $GTP\gamma S$) specifically. Thiophosphorylation was demonstrated with retinal rod outer segment membranes (Wieland *et al*., 1991) and phosphorylation was demonstrated subsequently with HL-60 membranes (Wieland *et al*., 1993). Sensitivity of the incorporated phosphate to HCl, hydroxylamine, and heat, and stability in NaOH, are consistent with a phosphoramidate linkage, implying histidine as the site of phosphorylation. The enzyme catalyzing the phosphorylation has not been identified but may be a nucleoside diphosphate kinase (Klinker and Seifert, 1999). Thiophosphorylated $\beta\gamma$ can serve as an intermediate in the formation of $GTP\gamma S$ when presented with GDP, which might explain, if the $GTP\gamma S$ subsequently binds to $G\alpha$ subunits, why it would decrease high-affinity fMet-Leu-Phe binding to HL60 membranes (Wieland *et al*., 1991) or alternately stimulate or inhibit adenylyl cyclase in platelet membranes (Wieland *et al*., 1992). The implication that the $GTP\gamma S$ so formed acts via $G\alpha$ subunits, however, has been challenged (Hohenegger *et al*., 1996).

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